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This grant is aimed at identifying tumor suppressor genes, one of the major goals of contemporary cancer research. A promising new approach in these efforts is to take advantage of the fact that growth regulatory genes can be easily identified in the fruitfly *Drosophila* and that such genes are highly conserved in evolution. We have found a novel gene in *Drosophila, tartaruga* (*tar*) that is involved in repressing target genes of an important signaling pathway, the Wnt pathway. The *tar* gene is highly conserved and this grant addresses whether the mammalian homologs of *tar* are involved in cancer. We have characterized a mouse homolog of *tar* at the molecular level. We also found a second homolog of the gene in mouse and human DNA. We are in the process of generating mice with constitutive or conditional-loss-of function mutations in the gene. Constructs for these gene targeting experiments have been built. We have also mapped the human *tar* gene (s) on the genome.

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INTRODUCTION

The goal of this grant has been to clone, map, and characterize the mammalian *tartaruga (tar)* gene in order to understand more about its function in cancer and, secondarily, in development. Our approach in the mouse has been to study expression at the RNA level, and to map the genomic structure in order to generate a knock out mouse for examination of the loss of function phenotype. Once the mouse is derived, it will be exposed to carcinogenic agents in order to uncover tumor susceptibility. Our approach in the human has been to study the expression pattern using Northern blot analysis and, after mapping the gene, to examine the locus for linkage to known LOH regions.

BODY

Project 1: The mouse *tar* gene

Expression of the mouse *tar* gene at RNA level

To examine the expression of mouse *tar* at the RNA level, multiple tissue Northern blots from adult tissues as well as developmental Northern blots from whole embryos at days 7,11,15 and 17 were made or obtained commercially. The blots were examined with cDNA probes made from IMAGE clones identified in a BLAST search with the conserved regions of *Drosophila tartaruga.* Additionally, frozen sections of adult organs were examined by in situ hybridization using riboprobes made from the same clones. We learned that mouse *tartaruga* is expressed at the earliest day examined, that it is expressed in all tissues examined, and that it produces three transcripts that are 8.5, 6.5, and 6.0 kb in length. After cloning the gene (below), we used RT-PCR to examine RNA from a variety of tissues in order to discover transcript specificities, but our results were not consistent enough to draw conclusions.

Clone full-length *tar* cDNA

In order to clone the full length cDNA, we screened a day 14 embryonic cDNA library in which we knew *tartaruga* was highly expressed, based on the work above. The cDNA clones obtained contained a great deal of genomic DNA, but after sequencing over 40 clones, we were able to assemble a 3 kb cDNA contig for which every splice junction was confirmed by at least one spliced clone. We found that the open reading frame, which coded for a 615 aa protein, had alternate 3' ends after the most 3' conserved region ended, explaining two of the three transcripts observed. We did not find the 3' poly A signal, so it is possible that alternate 3' UTR lengths explain the third transcript. We then probed the Northern blots with distinct regions of the ORF, but did not identify any more differences in the three transcripts. Importantly, we found that the 5' conserved leucine zipper was present in all three transcripts. In October 1999, a matching 500 amino acid human sequence translated from a single cDNA was submitted to the GenBank databases, confirming our results. We are still not certain that we have the start site as there is a highly GC rich region 5' of what we believe is the start codon, making it difficult to examine the upstream region; however, even less additional 5' sequence has been found in the human gene.

Clone genomic *tar* and map gene structure

In order to obtain genomic clones of *tartaruga*, a 129SV BAC library was screened, and 6 positive BACs were obtained. Using primers to the known exons, introns were amplified by

long range PCR in order to learn intron lengths. These amplimers were also partially sequenced in order to identify splice junctions and develop an appropriate knock out strategy.

Clone full-length second *tar* gene and study expression

When examining human cDNA clones, we discovered that there was a second mammalian homolog of *tartaruga*. Because the two human paralogs were highly conserved, it seemed likely that both were functional, and that there probably was a mouse paralog also. Because a second mouse *tartaruga* could obscure the phenotype of the first, we realized we would have to clone the second gene if it existed and knock it out also.

By searching the GenBank mouse EST databases with short pieces of the 5' region of the human paralog, we were able to find an IMAGE clone containing a short (24 nucleotides) homologous region; however, the clone itself had been lost by the supplier. Using the sequence, we designed a primer that allowed us to amplify the intervening diagnostic zinc finger region by RT-PCR from mouse RNA, establishing that the second gene existed. We were then able to locate ESTs based on the extensive (2kb) 3' UTR , eventually finding an IMAGE clone long enough to contain half of the ORF. By alternately using human and mouse sequences to search the EST and genomic databases we were able to assemble a 4.2kb contig and have a cDNA clone that covers most of this region. Our expression studies of the second *tartaruga* were less extensive, consisting of Northern blot analysis only. As with *tartaruga 1*, there were three transcripts. In this case, there were no alternate 3' ends, but there were 2 different poly A signals.

Clone second genomic *tar* and map gene structure

We screened the BAC library and obtained 5 positive clones, then mapped the gene structure as with *tartaruga 1*. Before we were through, however, the mouse genomic sequence (C57BL strain) for this gene starting 3kb 5' of what we believe is the second exon was deposited in Genbank. We have confirmed the published structure and relevant restriction sites with our BACs, which come from strain 129, the source strain for the ES cells we are using.

Generate *tar* knockout constructs

The *tartaruga* proteins have two highly conserved regions, a 5' leucine zipper and a 3' zinc finger with very conserved flanking arms. In both genes, the second exon, just prior to the leucine zipper, which is contained in the third exon, has a non-unit number of codons. Therefore, it is possible to remove the first two exons, thus removing the leucine zipper altogether, and simultaneously introduce a frameshift that cannot be corrected by any possible combination of the remaining exons.

We have extensively restriction mapped the genomic DNA of both genes and have designed but not tested assays for recognition of correctly recombined ES cells. We have developed a cloning strategy in which the 3' and 5' arms are being cut from the BACs and placed into a replacement vector containing *pgk thymidine kinase* and *pgk neo* selection markers The *pgkneo* marker is flanked by loxP sites which can be removed by transient Cre-recombinase expression after homologous recombination at the ES cell level. At present both conventional constructs are partially built. The strategy was designed so that with minor modifications, the reagents being generated can also be used to build conditional knock-out constructs.

Project 2: The human *tar* gene

Study expression pattern

Probes made from human cDNA IMAGE clones were used to examine human multiple tissue Northern blots. As with mouse *tartaruga 1,* there were three widely expressed transcripts of lengths almost identical to those of the mouse. The second *tartaruga* gene was also found to produce three transcripts.

Map human tar genes on chromosomes

When looking for mouse *tartaruga* homologs using Southern blot analysis, we discovered a *Bgl II* polymorphism between the *C57BL/6J* and *M. spretus* strains. We used this polymorphism to genotype the Jackson Laboratory Backcross DNA mapping DNA panels, thus mapping *tartaruga 1* to within 0.5 cM of the Npr1 locus of Chromosome 3. By synteny, we had mapped the human *tartaruga* to human Chromosome 1, band q21-q22.

In order to map *tartaruga 2*, we searched the genome databases with additional sequence we had obtained by sequencing *tartaruga 2* IMAGE clones and identified a sequence tagged site that had been mapped four times. *Tartaruga 2* was thus mapped to Chromosome 19, interval D195899-D195407.

We did not find obvious candidate tumor suppressor genes or linkage with LOH regions at either of these loci.

KEY RESEARCH ACCOMPLISHMENTS

- Cloning mouse homologs of tartaruga
- Establising genomic organization genes
- Cloning and characterizing human homologs

REPORTABLE OUTCOMES

None

CONCLUSIONS

We are well on course to accomplish the goals of the grant. During year one, we have made significant progress in our aim of generating loss of function mutations in the mouse tartaruga gene. We have also characterized a second mouse gene and two human homologs. We do not expect major difficulties in achieving the ultimate goals.

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APPENDICES

None